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1. Operating Procedure

Here is the reminder of the iSperm testing procedures. Please read the distributor guideline and watch tutorial video for detailed procedure before reading this technical document and starting iSperm.

(1) Set up iSperm.

- I. Combine the case and your iPad.
- II. Check whether the light field of Microscopic Optical Lens Module is in the middle of the screen field.
- III. Check whether the light source is working.
- IV. Insert the heater over the optical lens sleeve. Connect the heater to the power until the LED indicator light starts twinkling.

(2) Set up iSperm App.

- I. Make sure the app on your iPad is the latest version.
- II. Turn on the video saving function. Though it is optional, the videos can help us clarify the problems when you encounter them.

(3) Semen preparation.

- I. Semen is emulsive and agglutinated easily, so make sure the semen sample is mixed evenly.
- II. Due to biomechanical properties, refrigerated semen should be kept at room temperature for at least 20 minutes before evaluation. Thus, make sure refrigerated sperm recover to the normal motile state.

(4) Sampling.

- I. Please keep the light source vertically when you dip the Base Chip in the center of the cup and push the Base Chip vertically into the Cover Chip.
- II. Remember to pair up base chip and cover chip before you insert the light source into the sleeve of the optical lens.
- III. The chips are paired up perfectly when there is a "click" sound. If the chips are paired up inadequately, the sample in the chip will drift and affect the result.

(5) Using iSperm App.

- I. Please place iPad mini on the desk with the screen facing up, holding iPad when analyzing will affect the result.
- II. The software uses video to analyze semen. Dirt, bubbles and drift

will affect the result.

- III. Please analyze the sample within 1 minute after sampling. Do not test the same chip many times. Because the space in the sampling chip is small and according to the biological properties, semen will agglutinate within 1 minute and affect the results.
- IV. If you want to compare two sets of iSperm, please use two sets of chips from the same sample. Do not try to test one set of chip with two iPads. The process of switching chips to another iPad will cause unpredictable error.

2. The principle of iSperm

Concentration:

The process of total sperm algorithm and motile sperm algorithm are described in motility section. Here we discuss the adjustment of the concentration algorithm of iSperm. There are three concerns when we adjust the concentration algorithm.

- (1) The distribution of sample concentrations.
- (2) The number of chips.
- (3) The linear relationship of different concentration.

Mini1_iOS 9.3		iSperm Swine (4.0.26)									
Estimated Conc.	1158.00	772.00	579.00	386.00	231.60	115.80	77.20	57.90	38.60	23.16	
Number	1	2	3	4	5	6	7	8	9	10	
Chip 1	1130.42	766.61	528.89	387.82	227.23	109.19	76.91	57.58	39.56	24.60	
Chip 2	1116.83	729.97	542.14	382.66	217.05	116.20	77.88	55.69	35.87	21.34	
Chip 3	1115.23	767.60	552.25	356.86	226.38	105.16	72.59	52.04	36.70	23.13	
Chip 4	1109.71	712.16	552.08	329.83	219.82	115.90	78.65	55.39	40.18	26.37	
Chip 5	1315.23	727.32	577.09	366.04	233.12	110.07	77.30	57.17	37.33	22.44	
Makler Counter	1118.00	745.33	559.00	372.67	223.60	111.80	74.53	55.90	37.27	22.36	

Form. 1 The concentration data sheet

As shown in Form.1, take iSperm Swine for example. First, we will estimate the concentration of original semen by using iSperm. And we will dilute the original semen into 10 different concentrations (about 0.6-fold dilution) from 1158 to 23 million/ml.

Second, we dipped 5 chips in every concentration and recorded 1 video from each chip. Thus, we will get 50 videos in total (5 chips * 10 concentrations).

Then, we counted the lowest concentration by Makler cell-counting chamber because the lowest concentration is in the concentration accuracy interval of Makler cell-counting chamber.

We got 22.36 from Concentration Number 10 by Makler and we multiplied the dilution rate in order to know the exact concentration of these 10 concentrations.

After going through the process above, we will know the linear relationship between different concentration and sampling error of different chips.

These data help us doing the adjustment of the concentration algorithm.

Motility:

The process of counting total sperms and motile sperms are shown in Fig.1. The motile sperm algorithm firstly acquires frames from the sperm video. The rectangular region in the center is cropped from each frame for

counting sperms. After image processing, we filter sperm by their feature to count the labeled sperms from the binary image. The number of motile sperms is the difference between the previous frame and the current frame. Finally, the number of motile sperms is divided by the average of total sperms that is computed by summing up the total sperm numbers of each acquired frame and dividing this sum with the total duration of the frame acquisition.

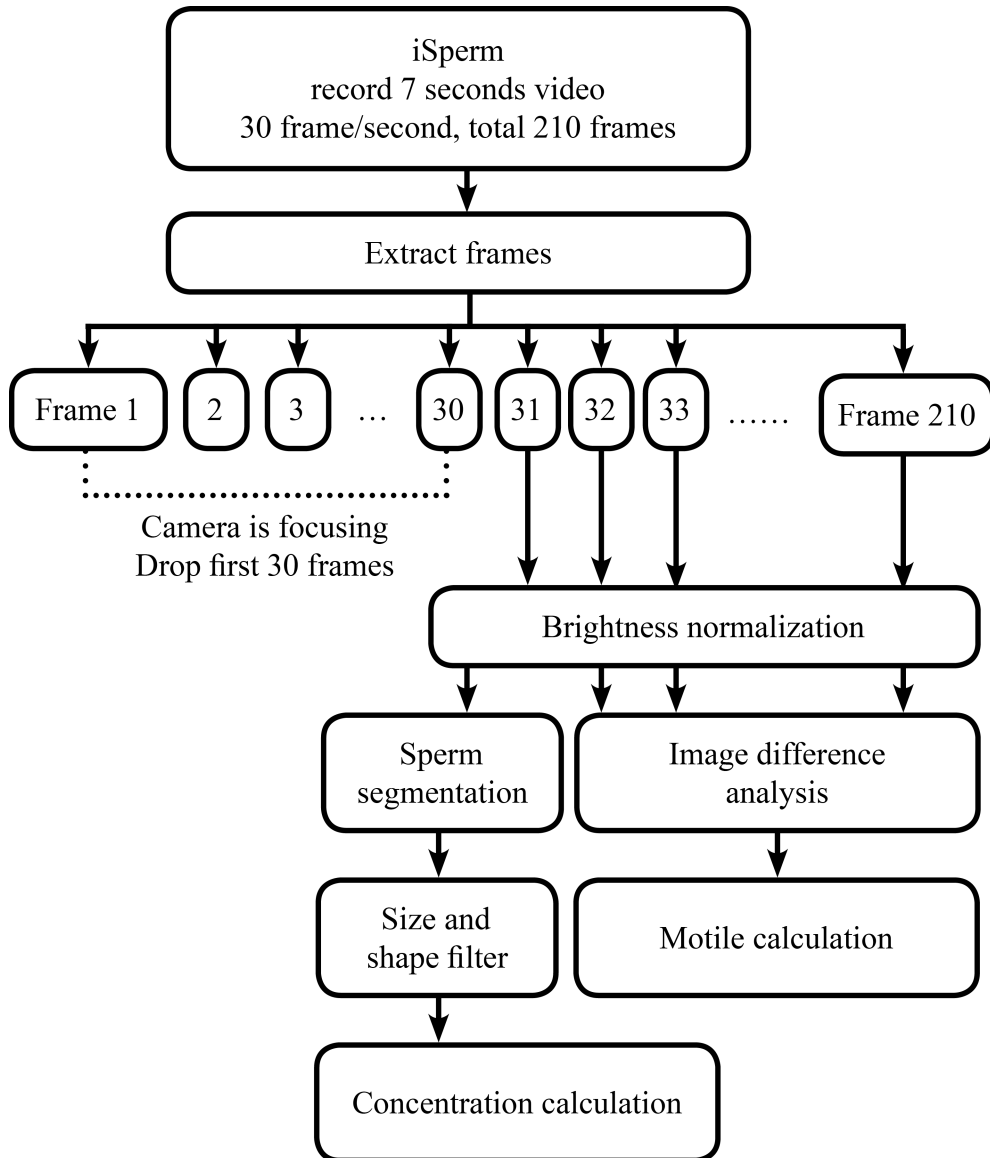


Fig.1 Flow chart of image processing for total and motile sperm algorithm

3. Photometer, CASA and iSperm

(1) The principle of photometer:

Photometer is an optical instrument for measuring the absorption of light of a given wavelength of colored substances in solution. From the light absorption, Beer's law makes it possible to calculate the concentration of the colored substance in the solution. The principle of spectrophotometers is that monochromatic light is allowed to pass through a container, which optically flat windows contain the solution. It then reaches a light detector, which measures the intensity of the light compared to the intensity after passing through an identical cell with the same solvent but without the colored substance. From the ratio between the light intensities, knowing the light absorbing capacity of the colored substance makes it possible to calculate the concentration of the substance using Beer's law.

This technology is using in semen quality measurement. Since the sample volume is about 20 μl , which is more than the sample volume of CASA and iSperm, its CV result is relatively low compare to CASA and iSperm. Moreover, it can only know the concentration of the semen. It still needs other equipment to measure sperm motility and their morphology.

(2) CASA System:

The results may vary among different CASA instruments (Hu et al., 2013); even within the same CASA system with consistent settings of parameters (Ehlers et al., 2011), it is therefore necessary to perform the standardization and quality control for CASA. There are some strict rules when using CASA system to measure semen quality. For example, sperm concentration should less than $50 \times 10^6 \text{ ml}^{-1}$. Also, the samples should be kept at the temperature of 37 °C. However, because there is no standard practice to follow for the current CASA system, and no associated detection standard for the CASA instrument to be recommended by professional associations, the CASA instruments themselves are very difficult to be standardised and quality controlled. Holt et al. (1994) evaluated the same semen sample using five different CASA systems and found that the variation within one CASA system was higher than that between other CASA systems for all detected

parameters, indicating that the variation in sample processing and the technician's skill was more pronounced than that of the CASA system itself and that the training for the technicians and the standardization of sample processing was going to increase the reproducibility of the CASA instrument. According to Holt's study, the average coefficients of variation (CV) for sperm concentration decreased from 26.1% to 11.3% ($P < 0.01$) and sperm motility from 5.8% to 3.1% ($P < 0.05$).

(3) iSperm:

According the operating procedure and algorithms described above, it is necessary to perform the standardization for iSperm. Moreover, the quality control should run through the entire process of semen analysis from the collection of semen samples to the detection result. Please follow the procedure described above to make sure the accuracy.

4. How to compare iSperm with other sperm quality evaluation tools

Please read the example of section 2.

If you want to inspect the concentration and motility of iSperm, here is the process that be suggested.

For fresh semen:

- (1) Collect fresh semen and remove impurities.
- (2) Warm up semen to 37°C with water bath.
- (3) Roughly estimate the semen concentration.
- (4) Dilute fresh semen with 0.6-fold dilution in to 5 to 10 different concentrations. For instance, in the roughly estimated semen concentration around 500 million/ml, you can dilute the semen into 500, 300, 180, 108, 64.8, 38.8 and 23.3 million/ml. A total of seven different concentrations of samples.
- (5) Dip 3 chips in every concentration and record 1 video from each chip. In the end you will get 21 videos (3 chips *7 concentrations).
- (6) Record the video names (IMG_00NN) and the corresponding iSperm results.
- (7) Take the sample of 23.3 million/ml to measure by CASA system.
- (8) Get the CASA system concentration and motility result and multiply the dilution rate to know the exact concentration of these 7 concentrations.
- (9) You will get a data sheet like this:

Roughly Estimated Conc.	iSperm Conc.	iSperm Motility	CASA Conc. (after multiply)	CASA Total Motility	Video File Names
500	480	80	512	77	IMG_0019

- (10) Now you will know the linear relationship between different concentrations and sampling error of different chips. Please provide the record in this format following the procedure indicated above whenever you find any problem after comparing iSperm with other sperm quality evaluation tools.

Since there are differences of concentration and semen volume between swine and other animals, you can adjust number of concentrations you dilute into. For example, if the original semen concentration of equine is about 350 million/ml, you can dilute semen into 6 different concentrations

from 350 to 20 million/ml. And you will get 18 videos. (3 chips *6 concentrations).

The videos recorded by iSperm are very important for us to understand the problems when you encounter them.

5. Reference

- (1) Holt W, Watson P, Curry M, Holt C (1994) Reproducibility of computer-aided semen analysis: comparison of five different systems used in a practical workshop. *Fertil Steril* 62:1277–1282.
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- (4) Fischer D, Neumann D, Wehrend A, Lierz M. (2014) Comparison of conventional and computer-assisted semen analysis in cockatiels (*Nymphicus hollandicus*) and evaluation of different insemination dosages for artificial insemination. *Theriogenology* 82:613–620
- (5) Wikipedia: <https://en.wikipedia.org/wiki/Photometer>